REGULATORY EFFECTS OF ADENOSINE DIPHOSPHATE ON THE ACTIVITY OF THE PLASMA MEMBRANE ATPase OF CORN ROOTS

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Received July 5, 1984

Plasma membrane enriched microsomal fraction was isolated from corn root cells by sucrose density centrifugation. The ATPase activity as measured by the release rate of inorganic phosphate, was decreased by the presence of modifiers which included diethylstilbestrol, vanadate, N,N'-dicyclohexylcarbodiimide, and miconazole. The presence of ADP also decreased the rate of ATP hydrolysis. Furthermore, a preincubation of the membrane with ADP significicantly reduced the inhibitory effects of these membrane ATPase modifiers. Since the modes of interaction of these modifiers with the enzyme are different, the results suggest that the binding of ADP may stabilize the plasma membrane ATPase in a modifier insensitive state.

It has been found that plant root cells contain a plasma membrane ATPase (1-3) which has an absolute specificity for Mg-ATP as substrate ($K_m \simeq 5 \times 10^{-4}$M). The properties of this enzyme, in many regards, are similar to that of fungal plasma membrane H^+-ATPase. A detailed comparison of these two enzyme systems was recently published (4). Both enzymes are distinctly different from F_0F_1-ATPases of mitochondria, chloroplasts, and bacteria (5). Rather, the enzymes share striking similarities with E_1E_2-type ion translocating ATPases found in animal cells. Both the fungal (5,6) and plant root (7-10) plasma membrane ATPases contain a 100K-Da polypeptide which forms a phosphorylated intermediate in the catalysis of ATP hydrolysis. The highly purified fungal plasma membrane ATPase (11) and the partially purified root plasma membrane ATPase (12) have been reconstituted into phospholipid vesicles. The reconstituted systems exhibit an ATP hydrolysis-supported electrogenic proton pumping

[1] U. S. Department of Agriculture, Agricultural Research Service.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; DCCD, N,N'-dicyclohexylcarbodiimide; DES, diethystilbestrol; DTT, dithioerythritol. EDTA, ethylenediaminetetraacetic acid; MES, 2[N-morpholino]-ethanesulfonic acid.

activity. These results support the suggestion that plant root plasma membrane ATPase may play a central role in the transport processes between soil-root interface.

In the present work, we studied the sensitivity of ATPase to inhibitors in the presence and absence of ADP. The results suggest that the binding of ADP significantly decreases the effects of inhibitors on the plasma membrane ATPase activity of corn roots.

MATERIALS AND METHODS

Isolation of Plasma Membrane: Plasma membrane enriched microsomal fraction was isolated from corn roots according to a procedure described by Leonard and Hotchkiss (14) with appropriate modification. Untreated corn seeds (FRB-73, Illinois Foundation Seeds[2]) were germinated over filter paper saturated with 0.1 mM $CaCl_2$ in glass trays for 3 days at 28 °C in an Isotemp 300 series incubator. The roots were homogenized in a solution containing 0.30M sucrose, 5mM EDTA, 10mM DTT, and 0.1M Hepes at pH 7.75. The homogenate was then filtered through cheesecloth and centrifuged at 13,000 g for 15 minutes. The supernatant was further centrifuged at 87,000 g for 40 minutes. The crude microsomal pellet was dispersed with a glass rod and then resuspended in a small volume of the solution mentioned above and then layered over a discontinuous sucrose gradient which consisted of 14 mL of 40% sucrose and 22 mL of 31% sucrose solution. Further centrifugation at 25,000 rpm with a Beckman SW-28 rotor yielded the plasma membrane fraction at the interface of 31 and 40% layers. All the isolation procedures were performed at 4 °C. The protein content was determined by the biuret method. The obtained plasma membrane fraction was frozen in liquid nitrogen and stored at -20 °C until use.

Hydrolysis of Adenine Nucleotides: The hydrolysis of ATP and ADP catalyzed by plasma membrane at ambient temperature (24 °C) was measured by the appearance of inorganic phosphate in 2 mL of a solution containing 30 mM MES pH 6.3, 50 mM KCl, 5 mM $MgCl_2$, 50 μg membrane protein, 4 μg oligomycin, and other additions as detailed in Figures and Tables. The reaction was started by the addition of nucleotides and terminated by the addition of 2 mL of ice cold 5% trichloroacetic acid. The amount of inorganic phosphate (P_i) formed was determined by the malachite green molybdate complex method as previously described (15).

Origin of Phosphate Released: With both ADP and ATP present, the percentage of the total P_i released from the latter was determined by the use of [γ-^{32}P]-ATP. A small aliquot of the reaction mixture was applied to a polyethyleneimine (PEI) treated cellulose thin layer plate of Brinkman. The plate was developed with a mixture of 1.8 M HCOOH and 0.2 M LiCl and the spots of nucleotides and P_i were located and removed. The radioactivity of these fractions were then measured with a Beckman LS-300 scintillation counter.

Chemicals: Oligomycin, DES, miconazole, ADP, ATP, Hepes, sucrose (RNAase free), and malachite green were obtained from Sigma Co. DCCD (gold label) was pruchased from Aldrich. [γ-^{32}P]-ATP was a product of New England Nuclear. All other chemicals used were of analytical grade.

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[2] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

RESULTS

Hydrolysis of Adenine Nucleotides: The ATPase activity of the plasma membrane used in this study was not inhibited by oligomycin (80 μg/mg of protein), indicating the absence of mitochondrial ATPase activity under the experimental conditions. In agreement with a previous report (14), the corn root plasma membrane enriched fraction catalyzed the hydrolysis of ATP and ADP. It has been shown that the ATPase activity may be described by simple Michaleis Menten kinetics (7,14). As shown in Figure 1, the hydrolysis of ADP in the presence of the membrane may also be described by these kinetics. Under the present experimental conditions, both the K_M and V_{max} of the ADP hydrolysis (0.31 mM and 9.90 μmol P_i/mg. hour) are similar to that of the ATPase activity (0.20 mM and 8.55 μmol P_i/mg. hour). Although the origin of this ADP hydrolysis is unknown, we shall refer to this activity as ADPase of the plasma membrane only for convenience. The membrane preparation used did not catalyze the hydrolysis of AMP and did not contain any adenylate kinase activity (data not shown).

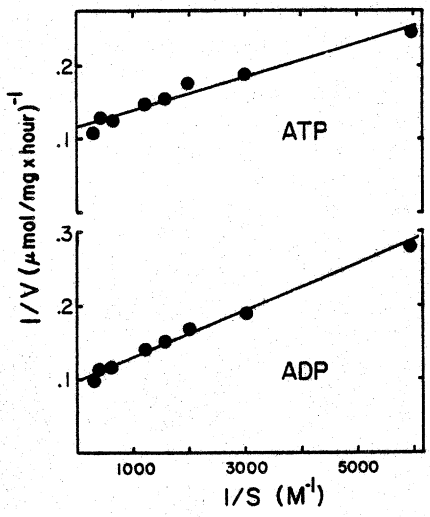

Figure 1. Kinetics of ATP and ADP Hydrolysis. The hydrolysis of adenosine phosphate catalyzed by the membrane was measured by the release of P_i in the medium as mentioned in Material and Methods. The hydrolysis was allowed to proceed for 10 to 20 minutes to limit the extent of nucleotide hydrolysis to be less than 10%. Thus, the average rates measured were close to the true initial rates. The double reciprocal plots of 3 independent measurements yielded K_M and V_{max} as 0.201 mM and 8.55 μmol P_i/mg.hr for ATP hydrolysis, and 0.307 mM and 9.90 μmol P_i/mg.hr for ADP hydrolysis.

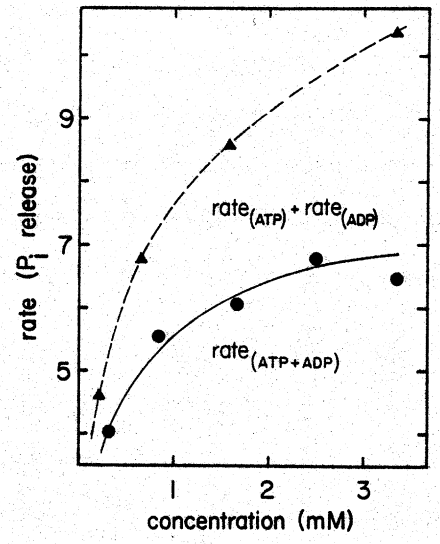

Figure 2. Non-additivity of ATPase and ADPase. The total P_i release rate with both ATP and ADP present simutaneously (molar ratio = 1.0), was compared to the sum of rates obtained with individual substrate of the same concentration as present in the solution of mixed nucleotides. The average hydrolysis rates obtained in the presence of mixed nucleotides and the calculated sum of rates obtained with individual substrate are shown as ———— and — — — — — —, respectively. The membrane used for each experiment contained 50 μg of protein.

Non-additivity of ATPase and ADPase: Although the membrane preparation catalyzed the hydrolysis of both ADP and ATP, these two activities were not additive. This non-additive effect is shown in Figure 2. The similar kinetic properties and this non-additive effect seem to suggest that the two nucleotides may compete for the same catalytic site(s). In order to obtain more quantitative information, the hydrolysis of ATP in the presence of ADP was determined by the use of [γ-^{32}P]-ATP. As shown in Table I, only the hydrolysis of ATP is substantially inhibited by the presence of ADP. This inhibition is consistent with the general reaction mechanism of E_1E_2-type membrane ATPases (16) which show competitive inhibition by ADP, a product of the reaction. It was found that this inhibition decreased as the concentration of ADP decreased. However, the presence of the ADPase activity prevented us from determining the K_I of ADP.

Responses to the Presence of Modifiers: The inhibition of ATPase by DES, DCCD, and vanadate in detergent treated root plasma membrane has been studied (4). In addition to these modifiers, as shown in Figure 3, we have found that miconazole is also a very effective inhibitor to the ATPase activity of untreated

TABLE I Origin of Phosphate Released[1]

Experiment	Total Phosphate	ATP Hydrolysis	ADP Hydrolysis
#1. 3.3 mM ADP[2]	266 nmol	0	266 nmol
#2. 3.3 mM ATP[2]	503 nmol	503 nmol	0
#3. (3.3 mM ADP + 3.3 mM ATP)[3]	412 nmol	157 nmol	255 nmol
#4. (3.3 mM ADP + 3.3 mM ATP + 4.5×10^{-5} M DES)[3]	337 nmol	94 nmol	243 nmol

1 Average of two measurements. Estimated error = ±10 %.
2 The hydrolysis of ADP or ATP catalyzed by membrane containing 50 μg of protein in the assaying medium for 1 hour was determined by the colorimetric method mentioned. The total amount of nucleotide used was 7400 nmols.
3 [γ-^{32}P]-ATP was mixed with ADP before adding to the assaying solution. The P_i released from ATP was determined from radioactivity counting as described in Material and Methods. The P_i released from ADP was calculated by substracting ATP hydrolysis from the total P_i release which was determined colorimetrically.

membrane preparation. However, the ADPase activity of the membrane was not significantly affected by these modifiers. For example, the presence of 1.8×10^{-5}M DES, 1.7×10^{-5}M vanadate, 4.1×10^{-5}M DCCD, or 2.5×10^{-4}M miconazole inhibited 50% of the ATPase activity but showed no effects on the

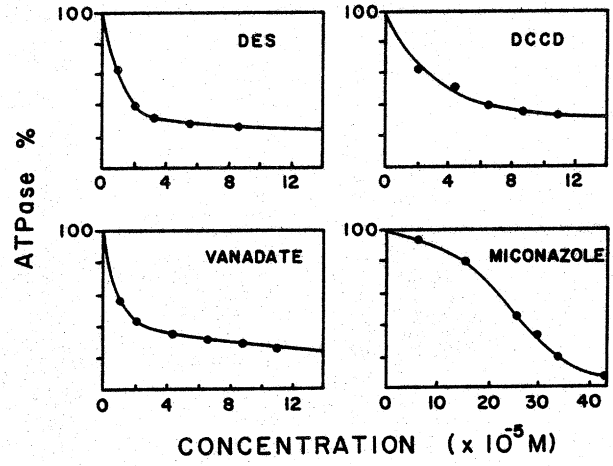

Figure 3. Inhibition of ATPase Activity. The membrane was allowed to be treated with the modifiers in the assaying medium for about 10 minutes before the addition of ATP (3 mM). The average P_i release rate was then determined as mentioned. Freshly prepared modifiers, with the exception of vanadate, were dissolved in ethanol or dimethylsulfoxide before adding to the medium. The final concentration of organic solvent in each sample, including the control, was about 2 % by volume. This level of organic solvents was found to have negligible effects on ATPase activity under the employed experimental conditions.

hydrolysis of ADP (data not shown).. As shown in Table I (experiment #4), the ADPase activity remained unaffected by ATP when DES was also included. These data seem to suggest that the ADPase activity does not originate from the same catalytic site(s) of the plasma membrane ATPase. In contrast, the hydrolysis of both ADP and ATP by a microsomal membrane preparation from pea stems exhibit parallel inhibition by DES and vanadate (17).

Sensitivity of ATPase Activity to Modifiers in the Presence of ADP: In order to test whether the inhibition of ATPase activity by modifiers might be affected by the presence of ADP or not, the experiments mentioned in Table II were performed. When the modifiers were added before the ADP incubation, no apparent protection of ATPase activity was observed. However, a brief incubation of the membrane with ADP before the addition of modifiers significantly decreased the inhibitory effects to the ATPase activity. The use of AMP, instead of ADP at the same concentration level, did not affect the normal inhibition of ATPase activity by the modifiers (data not shown).

TABLE II. Effects of ADP Preincubation on the Inhibition of ATPase[1]

Modifier used	Relative ATPase Activity	
	+ ADP Preincubation[2]	- ADP Preincubation[3]
none	100 % (5.26)	100 % (8.21)
4.8×10^{-5}M DES	65 % (3.42)	31 % (2.55)
4.8×10^{-5}M vanadate	70 % (3.68)	34 % (2.79)
4.8×10^{-5}M DCCD	85 % (4.47)	47 % (3.86)
4.5×10^{-4}M Miconazole	55 % (2.89)	10 % (0.82)

1 Average of two measurements. Estimated error = ±5 %.
2 The membrane was incubated with 1.67 mM of ADP for 1 minute in assaying media before the addition of modifiers. Following the incubation with the modifiers for 20 seconds, [γ-^{32}P]-ATP was added (final concentration = 1.67 mM). The hydrolysis was terminated 30 minutes later. The ADP hydrolysis before the addition of ATP was sbstracted to calculate the total P_i release. The % of P_i originated from ATP hydrolysis was determined by the use of [γ-^{32}P]-ATP as mentioned.
3 The membrane was incubated with the modifiers for 20 seconds before the addition of ATP (final concentration = 1.67 mM). The rate of P_i release was determined colorimetrically.
The numbers in parentheses represent the actual observed ATPase activity in unit of μmol P_i released / mg. hour.

DISCUSSION

It is well known that ADP plays an important regulatory role in the function of F_0F_1-type of ATPases (18). For E_1E_2-type of ATPases, the general reaction mechanism suggests a product inhibition of ADP on the hydrolysis of ATP (16). However, it was also demonstrated that the binding of ADP to fungal plasma membrane ATPase also provides a more effective protection against the inhibition of N-ethylmaleiimide (13).

In the present study, the observed decrease of ATP hydrolysis rate by ADP supports the idea that the plasma membrane ATPase of corn root may be an E_1E_2-type of ATPases which normally show product inhibition. The origin of the apparent protective effects of ADP on the ATPase activity against the inhibition of covalent and noncovalent modifiers used in this study, is presently unknown. However, it seems to be reasonable to suggest that the binding of ADP may stabilize the conformation of the active site of the ATPase. The answer to the question of whether the product inhibition and the stabilization originate from the binding of ADP to the same site or not, awaits for the availability of purified root plasma membrane ATPase.

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